

CONCISE COMMUNICATION

Levels of Matrix Metalloproteinase–9 within Cerebrospinal Fluid in a Rabbit Model of Coccidioidal Meningitis and Vasculitis

Paul L. Williams,^{1,2,a} Stephen L. Leib,⁵
Perparim Kamberi,^{2,3} David Leppert,⁶
Raymond A. Sobel,^{3,4} Yoeng-Delphine Bifrare,⁵
Karl V. Clemons,^{2,3} and David A. Stevens^{2,3}

¹Kaweah Delta Health Care District, Visalia, ²California Institute for Medical Research and Santa Clara Valley Medical Center, San Jose, ³Division of Infectious Diseases and Geographic Medicine, Department of Medicine, Stanford University, Stanford, and ⁴Veterans Affairs Medical Center, Palo Alto, California; ⁵Institute for Infectious Diseases, University of Bern, Bern, and ⁶Department of Research, University Hospital, Basel, Switzerland

Matrix metalloproteinase (MMP)–9 is produced by the central nervous system and inflammatory cells in a variety of inflammatory conditions in both animals and humans. MMP-9 promotes inflammation, breakdown of the blood-brain barrier, and vasculitis. Because vasculitis is seen frequently in patients with coccidioidal meningitis (CM), this study evaluated the presence of MMP-9 within the cerebrospinal fluid (CSF) of rabbits infected intracisternally with *Coccidioides immitis* arthroconidia. Infected rabbits demonstrated systemic and neurological sequelae to infection, including CSF pleocytosis. Levels of MMP-9 within CSF were assayed by use of zymography and compared with MMP-2 levels, which served as an internal control. Elevated levels of MMP-9 were detectable by day 3, continued to increase through day 10, and declined by day 15 after infection. MMP-9 may contribute to inflammation and vasculitis in this animal model. Future work can focus on evaluation of MMP inhibitors, to gain a better perspective of the role of this MMP in CM.

Matrix metalloproteinases (MMPs) are a family of endopeptidases produced by a variety of inflammatory cells [1]. In addition, MMPs are made by resident brain and vascular cells during central nervous system (CNS) infection [2]. These enzymes are thought to have a major role in promoting destructive inflammatory processes associated with infection, including disruption of the blood-brain barrier (BBB), edema formation, and vascular compromise, with resultant ischemic injury to neural tissue [2]. MMP-9 appears to play a pivotal role in the pathogenesis of adverse inflammatory processes, including vasculitis, as reported for human *Streptococcus pneumoniae* meningitis and corresponding animal models [2–4]. Coccidioidal meningitis is a highly lethal disease in humans, and vasculitis plays an important role in its morbidity and lethality, since, despite appropriate ther-

apy, up to 40% of patients exhibit this complication, as assessed by computed tomography scanning [5].

We sought to address the pathogenesis of meningeal inflammation and vasculitis by assessing the time course of changes in MMP-9 levels in cerebrospinal fluid (CSF) in a rabbit model of *Coccidioides immitis*-induced meningitis and vasculitis [6]. CSF levels of MMP-9 have not been addressed either in humans with coccidioidal meningitis or in an animal model of *C. immitis*-induced meningitis. In the present study, over a 2-week period we compared the CSF levels of MMP-9 in rabbits infected intracisternally with *C. immitis* arthroconidia with levels in uninfected rabbits that received intracisternal injections of sterile saline.

Methods

Preparation of *C. immitis* arthroconidia. Arthroconidia of *C. immitis* for intracisternal injection were prepared to a final concentration of 3.2×10^4 arthroconidia/0.25 mL, as described elsewhere [5].

Animal inoculation and immunosuppression. Five New Zealand white male rabbits (Myrtle's Rabbitry) weighing ~2.5 kg were anesthetized as described elsewhere [5] and received, in a class II biologic safety cabinet, an intracisternal injection of 3.2×10^4 arthroconidia. Five matched control rabbits were injected in a similar fashion with 0.25 mL of sterile saline. All rabbits were given 2.5 mg/kg Solu-Cortef (Steris Laboratories) intramuscularly, beginning the day before infection, the day of infection, and for 3 consecutive days after infection, as described elsewhere [5].

Postinfection CNS sampling and monitoring. On days 3, 7, 10,

Received 20 March 2002; revised 16 July 2002; electronically published 1 November 2002.

Presented in part: 41st Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, 16 December 2001 (abstract J471).

Financial support: Community Outreach Funds (grant to Kaweah Delta Health Care District); Swiss National Science Foundation (grant 32-61654.00); National Institutes of Health (grant NS-35902); Meningitis Research Foundation; Bank of Stockton (Stockton, California).

^a Present affiliation: Kaiser Permanente, Fresno, California.

Reprints or correspondence: Dr. Paul L. Williams, Kaiser Permanente, Dept. of Medicine, 7300 N. Fresno St., Fresno, CA 93720 (Pwilliam@pol.net).

The Journal of Infectious Diseases 2002;186:1692–5

© 2002 by the Infectious Diseases Society of America. All rights reserved.
0022-1899/2002/18611-0023\$15.00

and 15 after infection, animals were anesthetized with isoflurane gas, and ~0.25–0.5 mL of CSF was removed by cisternal puncture under sterile conditions. After white blood cell count analysis by hemocytometer, the remaining CSF was immediately centrifuged and frozen at -80°C until processed for MMP-9 analysis by electrophoresis and zymography. Rabbits were monitored twice daily for neurological complications and clinical signs. On day 15, all rabbits were anesthetized, CSF and blood samples were taken, and rabbits subsequently were killed with sodium pentobarbital (Euthasol; Delmarva Laboratories) given intravenously. Brains, spinal cords, and brain basilar arteries of infected and control rabbits were removed and fixed in 10% neutral buffered formalin for subsequent histologic examination.

Measurement of MMP-2 and MMP-9 levels in CSF. Samples of CSF (18 μL) were diluted into sample buffer (0.4 M Tris-HCl [pH 6.8], 10% SDS, 34% glycerol, and 1% bromphenol blue) to a loading volume of 24 μL and electrophoresed under nonreducing conditions in 10% polyacrylamide-SDS gels containing type A gelatin (1 mg/mL) as proteinase substrate [6]. After electrophoresis for 2.5 h at 95 V, MMPs were renatured by removal of SDS by bathing the gel for 1 h in Triton X-100 (2.5% vol/vol). Gels were then incubated in 10 mM CaCl_2 , 50 mM Tris, and 50 mM NaCl (pH 7.65) for 18 h at 37°C , to allow proteolysis of the gelatin substrate, were fixed, and were stained with Coomassie blue. The gelatinolytic activity of MMP-9 and MMP-2 was determined by densitometric quantitation of gelatin lysis zones at 92 (MMP-9) and 72 (MMP-2) kDa, using the Image program (version 1.61; National Institutes of Health). Infection did not influence the concentration of the constitutively produced MMP-2 in CSF of animals (measured at days 0, 3, 7, 10, and 15), compared with uninfected control animals. The amount of induced MMP-9 protein was expressed as a percentage of the amount of MMP-2, which was used as an internal standard. During electrophoresis, MMPs, even if present as proenzyme or in the active form but linked to their natural inhibitors, are activated by partial denaturation in SDS and/or by autoproteolysis with reconstitution of enzymatic activity (renaturation) occurring with Triton-X bathing [7]. Hence, the proteolytic bands for MMP-9 and MMP-2 reflect total levels of enzymes; however, this may not measure the enzymatic activity in vivo at that particular moment. Experiments to show the specificity of the assays for MMP-9 and MMP-2 were done by blocking these gelatinolytic zones by addition of β -aryl-succinic acid hydroxamate [8] and EDTA, 2 agents that specifically block these MMP enzymes in the incubation buffer (data not shown).

Histologic analysis. Histologic assessment of severity of meningitis was undertaken as described elsewhere [9].

Statistical analysis. Normally distributed variables were presented as mean \pm SD, and differences between groups were compared with the Mann-Whitney rank sum test.

Results

Clinical, neurological, and histopathological consequences of *C. immitis* infection were documented in all infected rabbits. All infected rabbits, but no control rabbits, demonstrated impairment in mobility, abnormal posture changes (reflecting menin-gismus), fever up to 40.2°C , and weight loss. CSF pleocytosis

was demonstrated in all infected rabbits beginning at day 7, whereas none of the control rabbits demonstrated pleocytosis. *C. immitis* was recovered from the CSF in all infected rabbits (≥ 20 cfu/ mm^3) beginning at day 7, and significant growth of *C. immitis* was also demonstrated from brain (mean, 3.23 \log_{10} cfu/g) and spinal cord cultures (mean, 2.8 \log_{10} cfu/g).

A representative zymogram, comparing day 7 MMP-9 levels in infected rabbits with those in control rabbits, is shown in figure 1. The time course of individual rabbit CSF assessment of the amount of MMP-9 for a 15-day period after infection or sham inoculation is presented in figure 2. One infected rabbit could not be tapped on day 10, and another infected rabbit met criteria for being killed on day 10 after tapping. Thus, one data point is missing for day 10, and another is missing for day 15. In addition, we were unable to tap one control rabbit on both days 10 and 15. Significant amounts of MMP-9 were observed on day 3 in the infected group, peaked at days 7 and 10, and slowly decreased thereafter. Low amounts of MMP-9 in control rabbits were observed on day 7, which persisted until day 15.

Histopathological study of infected rabbits showed evidence of meningitis of moderate or greater severity in 4 rabbits, with 1 rabbit demonstrating mild meningitis. No meningitis was observed in any of the control rabbits. Vasculitis involving meningeal vessels, brain basilar artery, or both was demonstrated in 4 rabbits. One rabbit did not demonstrate vasculitis but did demonstrate elevation of MMP-9 levels. Severity of meningeal inflammation and the presence of meningeal vasculitis, but not CSF white blood cell count, appeared to be associated with higher maximum levels of MMP-9 (table 1).

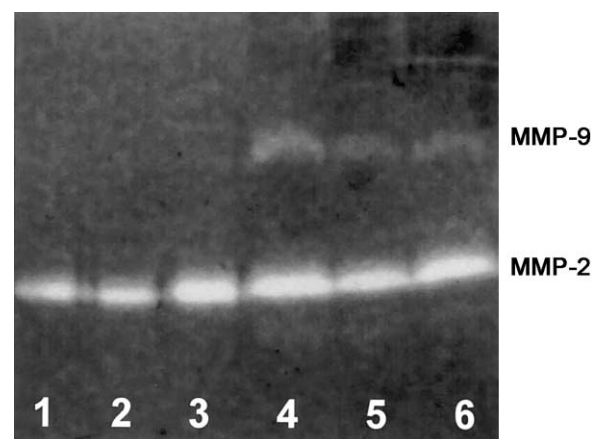


Figure 1. Representative zymography of cerebrospinal fluid (CSF) samples from uninfected control rabbits (lanes 1–3) and rabbits with coccidial meningitis (lanes 4–6) 7 days after infection. A gelatinolytic band of 72 kDa for the released matrix metalloproteinase (MMP)–2 was present in all samples, and there were no significant differences between uninfected and infected rabbits. CSF from infected rabbits showed a marked band at 92 kDa, indicating the presence of MMP-9.

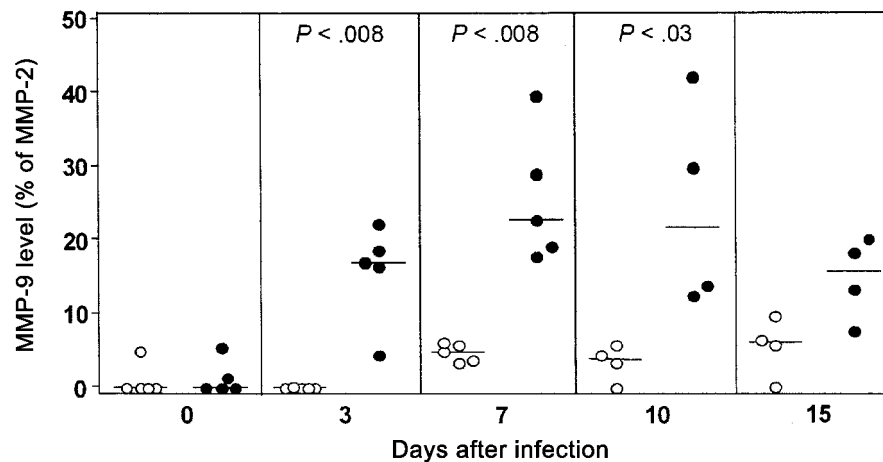


Figure 2. Matrix metalloproteinase (MMP)–9 concentration (expressed as a percentage of the constitutively released MMP-2) in cerebrospinal fluid of rabbits intracisternally infected with *Coccidioides immitis* (●) and uninfected control rabbits (○) over the course of 15 days. A significant increase in MMP-9 level was first detected at 3 days after infection, reached maximum levels at day 7, and persisted at significantly high levels until day 10. $P < .05$ was considered to be significant.

Discussion

Significant increases in MMP-9 levels were observed over a 2-week period in the 5 *C. immitis*-infected rabbits that developed clinical meningitis and vasculitis, compared with sham-treated control rabbits. We have previously assayed supernatant of the parasitic-phase, endospore-seeded *C. immitis* growth medium through time of endosporeulation and did not demonstrate MMP-9 production by *C. immitis* (authors' unpublished data). This observation is consistent with previous reports implicating inflammatory cells and resident brain cells, but not the infectious agents, as the source of MMP-9 during CNS infection [2, 10].

MMP-9 has been implicated in the disruption of the BBB during infection, leading to brain edema. This occurs by disruption of the subendothelial basement membrane via proteolytic

activity that degrades type IV and V collagens [3]. In addition, MMP-9 is implicated in stimulating the migration of activated inflammatory cells across the BBB, with resulting further damage to the barrier by cytokine products of these cells [2, 3]. MMP-9 has also been reported to up-regulate production of tumor necrosis factor (TNF)- α , a major proinflammatory cytokine [2, 3]. TNF- α can, in turn, positively feed back to inflammatory cells, enhancing their production of MMP-9 and, thus, perpetuating TNF- α production. In an animal model of pneumococcal meningitis, these events were closely correlated with the severity of inflammation, including vasculitis, leading to breakdown of vascular integrity, decreased blood flow, and eventual thrombosis of

Table 1. Severity of meningitis, presence or absence of meningeal and basilar artery vasculitis, and maximum cerebrospinal fluid (CSF) white blood cell (WBC) count, compared with production of matrix metalloproteinase (MMP)–9, in *Coccidioides immitis*-infected and control rabbits.

Group, rabbit	Severity of meningitis	Meningeal vasculitis	Basilar artery vasculitis	Maximum CSF WBC count (day)	Maximum MMP-9 concentration (day) ^a
Infected					
152 ^b	Moderate	+	–	300 (7)	39.58 (7)
153	Severe	+	+	1900 (10)	29.81 (10)
154	Severe	+	+	1400 (10)	42.14 (10)
155 ^c	Mild	–	–	1850 (10)	19 (7)
156	Mild-moderate	–	+	1550 (10)	17.8 (7)
Control					
147 ^d	None	–	–	0	6.17 (7)
148	None	–	–	0	5.58 (15)
149	None	–	–	0	9.47 (15)
150	None	–	–	0	5.75 (10)
151	None	–	–	0	6.35 (15)

NOTE. –, Absent; +, present.

^a Expressed as a percentage of the constitutively released MMP-2.

^b Unable to tap at day 10.

^c Killed at day 10 after tap.

^d Unable to tap at days 10 and 15.

vascular lumens. This ultimately leads to focal ischemic and infarction sequela. Ampel et al. [11] have demonstrated significant levels of TNF- α within the CSF of patients with coccidioidal meningitis.

MMP-9 also appears to play a pivotal role in enhancing inflammation within the meninges and within the brain parenchyma and vascular tree of humans with tuberculous, candidal, or streptococcal meningitis [3, 12]. In addition, Sorbi et al. [13], have demonstrated that MMP-9 is associated with the production of vasculitis in humans with temporal arteritis. Thus, MMP-9 activity appears to be a generalized CNS response to a number of inflammatory conditions.

Our protocol for establishing a rabbit model of coccidioidal meningitis and vasculitis calls for immunosuppressing rabbits during the first few days of infection. Immunosuppression could have affected the temporal events influencing MMP-9 levels within the CSF of infected animals (i.e., by slowing production) and may have affected the absolute levels produced. It would likely not have affected the final interpretation of results, however, since control animals were also treated with steroids in exactly the same fashion and had lower levels of MMP-9. The finding of small amounts of MMP-9 in the control group nonetheless is of interest and could signify the influence of environmental stress factors, anesthesia, and frequent intracisternal punctures in promoting production of MMP-9, possibly by direct effects on resident CNS cells.

It is of interest that the amount of MMP-9 demonstrated in this study may promote inflammation and vasculitis in our model. The increase of MMP-9 in the present rabbit model of coccidioidal meningitis is slower and less intense but far more protracted, compared with that in bacterial meningitis [2, 9] and, to a lesser extent, viral meningitis [1]. Hence, the longitudinal course of MMP-9 up-regulation is in accordance with the subacute clinical disease.

Our observations here enable the future study of MMP-9 inhibitors to assess the role of MMP-9 in promoting meningeal inflammation and vasculitis. Recently published data [2, 10] demonstrate that 2 inhibitors of MMP-9 (GM6001 and BB1101), when given intraperitoneally or subcutaneously, respectively, to rats with experimental *S. pneumoniae* meningitis, can block the activity of both MMP-9 and another metalloproteinase, TNF- α -converting enzyme, which correlated with decreasing severity of inflammation, an effect that could be used therapeutically in coccidioidal meningitis. Future studies with our rabbit model will examine the role of endogenous inhibitors of tissue inhibitors of metalloproteinases and whether other MMPs (such as MMP-8 and MMP-13) are up-regulated in coccidioidal meningitis.

Acknowledgments

We thank Katharine Fletcher for excellent secretarial assistance, David Hewitt (director of the Kaweah Delta District Hospital Laboratory, Visalia, California) for laboratory support, and Leilani Calderon for research assistance.

References

- Leppert D, Lindberg RL, Kappos L, Leib SL. Matrix metalloproteinases: multifunctional effectors of inflammation in multiple sclerosis and bacterial meningitis. *Brain Res Brain Res Rev* **2001**;36:249–57.
- Leib SL, Leppert D, Clements J, Täuber MG. Matrix metalloproteinases contribute to brain damage in experimental pneumococcal meningitis. *Infect Immun* **2000**;68:615–20.
- Leppert D, Leib SL, Grygar C, Miller KM, Schaad UB, Holländer GA. Matrix metalloproteinase (MMP)-8 and MMP-9 in cerebrospinal fluid during bacterial meningitis: association with blood brain-barrier damage and neurological sequelae. *Clin Infect Dis* **2000**;31:80–4.
- Azeh I, Mäder M, Smirnov A, Beuche W, Nau R, Weber F. Experimental pneumococcal meningitis in rabbits: the increase of matrix metalloproteinase-9 in cerebrospinal fluid correlates with leukocyte invasion. *Neurosci Lett* **1998**;256:127–30.
- Williams PL, Johnson R, Pappagianis D, et al. Vasculitic and encephalic complications associated with *Coccidioides immitis* infection of the central nervous system in humans: report of 10 cases and review. *Clin Infect Dis* **1992**;14:673–82.
- Williams PL, Sobel RA, Sorensen KN, et al. A model of coccidioidal meningoencephalitis and cerebrospinal vasculitis in the rabbit. *J Infect Dis* **1998**;178:1217–21.
- Leppert D, Waubant E, Galaray R, Bunnett NW, Houser SL. T cell gelatinases mediate basement membrane transmigration in vitro. *J Immun* **1995**;154:4379–89.
- Kottirsch G, Koch G, Feifel R, Newmann U. β -aryl-succinic acid hydroxamates as dual inhibitors of matrix metalloproteinases and tumor necrosis factor alpha converting enzymes. *J Med Chem* **2002**;45:2289–93.
- Sorensen KN, Sobel RA, Clemons KV, Pappagianis D, Stevens DA, Williams PL. Comparison of fluconazole and itraconazole in a rabbit model of coccidioidal meningitis. *Antimicrob Agents Chemother* **2000**;44:1512–7.
- Leib SL, Clements JM, Linberg RLP, et al. Inhibition of matrix metalloproteinases and tumor necrosis factor converting enzyme as adjuvant therapy in pneumococcal meningitis. *Brain* **2001**;124:1734–42.
- Ampel NM, Ahmann DR, Delgado, KL, Galgiani JN, Cloud GA. Tumor necrosis factor- α and interleukin-1 β in cerebrospinal fluid of patients with coccidioidal meningitis during therapy with fluconazole. The National Institute of Allergy and Infectious Diseases Mycosis Study Group. *J Infect Dis* **1995**;171:1675–8.
- Matsuura E, Umehara F, Hashiguchi T, Fugimoto N, Okada Y, Osame M. Marked increase in matrix metalloproteinase 9 in cerebrospinal fluid of patients with fungal or tuberculous meningeal encephalitis. *J Neurol Sci* **2000**;173:45–52.
- Sorbi D, French DL, Nuovo GJ, Kew RR, Arbeit LA, Gruber BL. Elevated levels of 92-kD type IV collagenase (matrix metalloproteinase 9) in giant cell arteritis. *Arthritis Rheum* **1996**;39:1747–53.